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Biodiversity and biotechnological potential of filamentous fungi isolated from Tunisian olive mill biotope

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Abstract

The increase in olive production and olive oil will generate much larger quantities of by-product that threaten the environment. Current trends should focus on the integration of various technologies to treat and valorize these effluents, but at a low cost. Therefore, it would be interesting to study the biodiversity of the filamentous fungi of olive by-products as well as their physiological and biochemical mechanisms. In this context, the aim of this work is to study biodiversity of filamentous fungi isolated from olive mill waste water and olive cake from different mills of Tunisia**.** The study of biodiversity of filamentous fungi was investigated through the isolation, purification and identification of new strains; and the study of the physiological and biochemical mechanisms of the selected strains by describing the nutritional needs and the metabolic potentialities of these microorganisms with a view to selecting strains capable of producing enzymes. A total of 47 strains of filamentous fungi were isolated and purified from samples obtained from the OMW and olive cake of different mills of Tunisia. The results obtained show that our fungi collection shows an important biotechnological potential, thereby the isolated strains can produce several extracellular enzymes of great interest for biotechnology and industry such as tannic acid, phytic acid, cellulase, amylase which represent high added value products. This study opens new uses of filamentous fungi present in olive mill waste water and olive cake to produce fungal enzymes, exploitable for the valorization of agricultural by-products.

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Introduction

Tunisia is the most important olive-growing country in the southern Mediterranean. If we exclude the European Union, Tunisia is the world's largest power in the olive oil sector, deploying major efforts to restructure, modernize and improve the quality of its oils, accompanied a considerable expansion of surfaces (Gharbi *et al.*, 2014). The average production of Tunisian olive oil is over the last 10 years (2006-2016) at 176 000 tones with a minimum of 70 000 tones and a maximum of 340 000 tones (ONH, 2016). The olive oil works an industrial fabric containing 1679 oil mills with a theoretical capacity of 43 053 tones/day (South 8%, Center 1.8%, sfax 25%, sahel 26% and north 23%). The exports of olive oil are the leading agricultural exports and represent 40% of all, in 2015 Tunisia exported 312,000 tons of olive oil out of the 340,000 produced (ONH, 2018). However, the increase in olive production and olive oil will generate much larger quantities of by-product that threaten the environment, especially olive mill waste water and olive cake (Nefzaoui, 1995). In this way, the pollution increases over the years and the search for an adequate treatment system becomes imperative (Benyahia and Zein, 2003). The different types of treatment currently used have been only able to partly solve this problem. Indeed, most of the proposed methods remain inefficient and even limited. Current trends should focus on the integration of various technologies to treat and valorize these effluents, but at a low cost (Hafidi *et al.*, 2005). This would therefore require the production of added value that will cover the extra costs generated by the application of these new technologies. Innovative biological processes, which exploit the metabolic flexibility of strains of filamentous fungi that naturally survive in olive mill waste water, olive cake and olives, remain par excellence the least costly processes, perfectly meeting of the problem posed in the olive sector. Thus as, the use of the method of beating, handling procedure during harvest of olives, storage in bulk or in bags, the extended stay of olives before their extraction of olive oil in olive mills offer the favorable conditions for the development of fungi on the olives, olive cake and olive mill waste water (Bavaro *et al.*, 2017).The fungi are a very heterogeneous group; the economic interest of filamentous fungi relies on their ability to produce a wide variety of molecules (Boiron, 1996). Thus, we find those who are harmful (biodeterioration, mycotoxins, allergies and mycoses) and useful (production of enzymes, antibiotics, organic acids, vitamins...). In addition, research on filamentous fungi has already acquired great interest, mainly in the production of enzymes used in industrial processes (Maheshwari *et al.*, 2000, Hassouni *et al.*, 2006 a, b). The fungi are characterized by high growth rates, which assumes that the biosynthesis of products derived from their metabolism would be particularly rapid (Cordova Lopez, 1998). Moreover, there are many studies suggest the existence of a correlation between the thermophilic of fungi and the thermostability of their proteins, especially their enzymes (Campos and Felix, 1995). The enzymes of thermophilic microorganisms grown at high temperatures can catalyze the biochemical reactions to high temperatures. In addition, this thermostability prolongs the storage life of enzymes (Dix and Webster, 1995 a, b, Jay 1996). The stability of biocatalysts is very important for use in industrial applications (Prenosil *et al.*, 1987). Among the enzymes, lipases, phytases; amylace, cellulase and tannase are as important enzymes in industry. However, the main obstacle hindering the applications potential of these enzymes is their cost. Therefore, it would be interesting to study the biodiversity of the filamentous fungi of olive by-products (olive cake and olive mill waste water) as well as their physiological and biochemical mechanisms by describing their nutritional needs.

In this context, we integrate our work, whohad a triple objective: first, to identify natural mycoflora present in olives mill waste water (OMW) and olive cake; then, the isolation, purification, description and identification of new strains from these specific biotopes; and last, the study of the physiological and biochemical mechanisms of the selected strains by describing the nutritional needs and the metabolic potentialities of these microorganisms with a view to selecting strains capable of producing enzymes.

Materials and methods

Samples origin

The sample of OMW and olive cake used in the present study were obtained from olive mills which uses a discontinuous process for extraction of olive oil, located in different regions of Tunisia (Al Krib, Téboursouk, Mornag, Gaâfour, Sfax, Zarzis, Châal, Jammel).

This region forms 3 groups from a geographic and especially climatic condition (Fig.1).

Fig. 1. The regions concerned by this study are mentioned in red: north (Teboursouk, Al Karib, Gaâfour, Mornag), Sahel (Jemmal) and south (Sfax, Châal, Zarzis).

The samples of OMW and olive cake were sampled during the olive growing season (November 2015-March 2016).

Microorganisms

Culture medium for strain isolation and identification

Potato dextrose agar (PDA) from Sigma (St. Louis, USA) was used for the isolation, purification, and conservation ofstrain(Botton *et al.*, 1990). For the identification of strain three culture media from Sigma were used according tostandard conditions: theMalt Extract Agar (MEA) medium, Czapeck agar medium (CZA), and Potato Dextrose Agar medium (PDA). Thesemedia were sterilized at 121°C for 20 min and distributed inPetri dishes. The initial pH was adjusted to 5.5.

Isolation strategy

ln order to isolate mesophilic strains present in olive mill waste water(OMW) and olive cake samples, the Petri dishes were incubated at 30°C for 72 h. So, after homogenization of the OMW samples, a series of dilutions in cascade in sterile distilled water is carried out. A volume of 0.1 ml of each appropriate dilution is spread on Petri dishes containing 20 ml of PDA medium.

For olive cake samples, three fragments were put at three different locations on the surface of Petri dishes containing 20 ml of PDA medium and incubated. Each sample was prepared in triplicate.

Isolation and conservation of strains

PDA, a nonselective medium, was used in the

purification steps. In the case of bacterial contamination, chloramphenicol (50 mg/L) was added (Botton *et al.*, 1990). The strains obtained in pure culture were kept on PDA at 4°C.

Identification of the strains

Morphological identification

To obtain data on the description and identification of the strains, cultures were grown on PDA medium, observed under a microscope for morphological characteristics and compared by reference to classical keys reported in the literature (Raper and Fennell, 1965; Barnet and Hunter, 1972; Ainsworth *et al.*, 1973;Arx, 1974). To obtain microscopic characterization of the cultures, superficial seeding was carried out on PDA in petri dishes at three points in the case of slow-growing fungi (*Aspergillus, Penicillium, Fusarium*)or at the center of the petri dish for fast-growing fungi (*Trichoderma, Rhizopus, Mucor*). Microculture techniques were used for characterization of mycelium and reproductive structures (Riddel, 1950). For each group of filamentous fungi, the strategy for identification was as follows:

Penicillium: for the standard microscopic description and Penicillium identification, classical references by Pitt (1979) and Samson *et al.* (1996) were used.

The techniques used by those authors consisted in inoculating the strains in three points on Petri dishes containing CZA or MEA at 2%. Then, cultures are incubated at30°C. Identification keys were then used.

Aspergillus: Identification keys proposed by Rapper and Fennell (1977) were used. The culture of Aspergillus was carried out on CZA with an incubation at 30°C.

Rhizopus: The determination of the Rhizopus species was carried out according to the key of Schipper (1978).

For other genera identification, keys by Cooney and Emerson (1964), Domsch *et al.* (1980), and Mouchacca (2000) were used.

Molecular identification

The morphological identification was followed by a molecular identification, which was carried out as follows: 1) Production of fungal biomass in culture medium (malt extract agar, MEA); 2) DNA extraction using the Genomic Prep Cells and Tissue DNA Isolation Kit (Biotools B&M Labs S.A.); and 3) PCR amplification of the ITS region from the rDNA [primers: ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4(5'TCCTCCGCTTATTGATATGC3')] so, 80 µl of reaction mixture contained 10 ng of template DNA, 10 mM of each ITS1 and ITS4 primer, PCR buffer 4 µl, 2.4 $MgCl_2$ (25 mM), 0.6 µl dNTPs (10 mM), 0.1 BSA (0.1mg/ml), 0.2 unit of *Taq*DNA polymerase and 8.7 µl d'H_2O_2, according to the protocol described by Attili *et al.* 1998.Theamplification conditions were as follows: an initial denaturing step of 3 min at 95 °C, followed by 45 cycles of95 °C for 60 s, 54 C for 60 s, and 72 °C for2 min, and then a final step at 72 °C for 7min. PCR reaction products were checkedfor length, quality and quantity by agarose gel electrophoresis and purification 1% (w/v) in 0.5% Tris Acetate-EDTA buffer (TAE).

Sequence analysis

Sequencing of all PCR products was carried out at Biotechnology and Chemometrics Team, St Jérôme Faculty; Aix Marseille University and at the private company Eurofins Genomics, Germany. Data were analyzed using the software Chromas and were compared to those in the 2015 GenBank database of NCBI [http://www.ncbi.nlm.nih.gov/] using the Basic Local Alignment Search Tool (BLAST) algorithm and the 2015 version of the tool Classifier of the Ribosomal Database Project [http://rdp.cme.msu.edu].

Analysis of the 16S rDNA sequence

The raw electropherograms in the Chromas® format were checked one by one. The pairs of sense /antisense sequences have undergone multiple and progressive alignment (Thompson *et al.*, 1994), with ClustalX® software, to verify the concordance of the data and establish a consensus sequence. The latter was submitted to Blast (Alschul *et al.*, 1997) to

establish the percentage identity of the strains studied with the closest species.

Physiological characterization of filamentous fungi

Determination of apical growth

The determination of the rate of apical growth for each strain reflects its ability to grow rapidly or not on a given medium at a defined temperature Smith and Berry (1975). It is evaluated by measuring the elongation of the mycelium in millimeters per hour (Roussos and Raimbault, 1982). from the center of the agar surface of the Petri dish (point of inoculation) to the periphery. This elongation is measured daily using a graduated ruler.

Relationship of apical growth:

Average speed: $Dmax/2/Time$

The average apical growth rate in mm/hour and Dmax the diameter in mm of the last day.

Sporulation index and spore production

Index of sporulation was determined as described by Roussos (1985) and expressed as the number of spores per gram of substrate utilized in the culture medium. Two to three drops of a spore suspension were inoculated into 250 ml Erlenmeyer flasks containing 50 ml of supercooled PDA and incubated at 30°C for 5-7 days.

The spores are recovered on the surface of the agar medium using an electromagnetic stirrer for about 30 minutes, in a Tween 80 solution (0.01%). For counting spores, we use the cell of Malassez under optical microscopy. For this purpose, a 1/100 dilution is carried out using water with tween. The count is valid when the number of spores is between 10 and 30 per reading field.

Nutritional study of fungi

Isolated strains were cultured on agar media containing different carbon sources (Table 1) such as proteins (casein), lipids (tween 80), starch, glucose, sucrose, carboxymethylcellulose (CMC), tannic acid (TA) and phytic acid (PA) to demonstrate their capabilities to develop on these carbon sources; and thereby indirectly screening the production of the extracellular enzymes.

Detection on solid medium of enzymatic activities

Phytic acid and tannase acid are characterized by the presence of a clear, visible haloon a black background. This corresponds to the hydrolysis zone of the appropriate substrate. For the other enzymes, they are highlighted by the growth of the fungus on the medium containing the corresponding substrate.

Results and discussion

Fungal isolates

40 olive mill waste water and olive cake samples were taken from different olive mills located in several areas of Tunisia (Fig. 1). After sampling, we began isolating mesophilic strains from OMW and olive cake.-Based on the isolation results, a mixture of fungi of different visual aspects was obtained for each Petri dish, also all the samples are loaded by the fungi but with dominance in the samples of the OMW compared to the olive cake. After purification, we first made a morphological identification to classify the strains of filamentous fungi within a genus or a species compared to reference strains.

Table 1. Compositions of culture media containing different carbon sources in g/l H_2O.

	Compositions of culture media (in g/l H_2O)													
Media	$CaCl_2$	$MgSO_4$	KH_2PO_4	NaCl	$(NH_4)SO_4$	Agar	Glu	Suc	Star	CMC	TAM	PAM	Cas	Tween
Glucose	0.48	0.38	2.47	0.32	6.6	20	10							
Sucrose	0.48	0.38	2.47	0.32	6.6	20		10						
Starch	0.48	0.38	2.47	0.32	6.6	20			10					
CMC	0.48	0.38	2.47	0.32	6.6	20				10				
TAM	0.48	0.38	2.47	0.32	6.6	20					4			
PAM	0.48	0.38	2.47	0.32	6.6	20						1		
Casein	0.48	0.38	2.47	0.32	6.6	20							10	
Tween	0.48	0.38	2.47	0.32	6.6	20								20

All nutritional media undergoing the same treatment except the tannic acid medium that undergoes sterilization by filtration.

After morphological identification we confirmed this by molecular characterization by PCR-sequencing. So, a total of 47 strains of filamentous fungi were isolated and purified from samples obtained from the OMW and olive cake of different mills of Tunisia, using different media at 30°C (Table 2).

Table 2. Different genera and species of fungi isolated from olive mill waste water and olive cake from different regions of Tunisia.

Origin / Region	OMW	Olive cake
Mornag	*Penicillium crustosum* *Geotrichumcandidum* *Mucor fragilis*	*Cladosporium cladosporioides* *Penicillium expansum*
Teboursouk	*Penicillium polonicum* *Penicillium commune* *Aspergillus tubingensis*	*Aspergillus pseudoglaucus* *Mucor circinelloides*
Gaâfour	*Trichoderma virens* *Penicillium commune* *Penicillium crustosum*	*Alternaria infectoria* *Fusarium oxysporum*
Al Karib	*Penicillium expansum* *Aspergillus awamori* *Aspergillus welwitschiae*	*Talaromycestrachyspermus* *Aureobasidiummelanogenum*
Zarzis	*Aspergillus proliferans* *Curvulariaspicifera* *Aspergillus ustus* *Aspergillus hiratsukae* *Penicillium griseofulvum*	*Talaromycesassiutensis* *Aspergillus niger* *Cladosporiumcladosporioides*
Sfax	*Aspergillus niger* *Penicillium polonicum* *Purpureocilliumlilacinum* *Aspergillus tubingensis*	*Geotrichumcandidum*
Châal	*Penicillium citreosulfuratum* *Aspergillus niger* *Aspergillus hiratsukae* *Aspergillus awamori* *Penicillium* *toxicariumMyceliophthoraverrucosa* *Penicillium commune*	*Aspergillus awamori* *Talaromycestrachyspermus*
Jemmal	*Alternariaalternata* *Rhizopusoryzae* *Aspergillus niger*	*Alternaria infectoria* *Aspergillus welwitschiae*

Identification of fungi

Aspergillus niger

On MEA and PDA medium is characterized by the thallus with fast apical growth at 30°C, aspect powdery white at first and completely black (hence niger) after sporulation (Fig. 2 A). The reverse is of ray color, with presence of wrinkles. Presence of exudate visible to the naked eye. Under the microscope we observe the mycelium is septate and presents a specific sporiferous form: head conidium

(aspergillate head) to uniseriate organization with only phialides or biseriate with the addition of the metules(Fig.2 B). The results of PCR and sequencing are presented in the Table 3 and Figure 3.

Rhizopus oryzae

The cultivation of *Rhizopus oryzae* strains on PDA is characterized by a very rapid apical growth of invasiveness, which can rise to the lid of the petri dish. The thallus has a woolly texture and first white and then gray color and brown gray lapel (Fig. 4 A). The mycelium is siphoned.

Table 3. Molecular and Morphological identification of *Aspergillus niger*.

Aspergillus sp		
Molecular identification	Accession number	Probability
Aspergillus niger	KX901281.1	100%
Morphological identification	*Aspergillus sp*	

Table 4. Molecular and Morphological identification of *Rhizopus oryzae*.

Rhizopusspp		
Molecular identification	Accession number	Probability
Rhizopus oryzae	MH865587.1	99%
Morphological identification	*Rhizopus spp*	

The hypha carries at its end the sporocyst which encloses the endospores (spores formed inside). Under the microscope, zygospores that do not exceed 50 µm in diameter. Globular spores, smooth and hyaline. Sporocyst echinulate and globular. Brown Sporocystophore, septate and very branched in sympods(Fig. 4 B).The results of PCR and sequencing are presented in the Table 4 and Figure 5.

Table 5. Molecular and Morphological identification of *Penicillium commune*.

Penicillium commune		
Molecular identification	Accession number	Probability
Penicillium commune	KC329623.1	99%
Identification morphologique	*Penicillium spp*	

Table 6. Fungal colony apical growth (mm/h) and sporulation index (spores/g carbon dry mater) of different strains incubated at 30°C during 7 days.

Strains	Apical growth	Sporulation index
G. candidum	0.27	$5.2\ 10^9$
A. tubingensis	0.19	$9.6\ 10^9$
M. fragilis	0.36	$6.2\ 10^9$
P. crustosum	0.15	$7.4\ 10^{10}$
A. infectoria	0.15	$1.85\ 10^9$
F.oxysporum	0.62	$2.4\ 10^9$
T.assiutensis	0.36	$6.2\ 10^9$
A. pseudoglaucus	0.29	$5.6\ 10^9$
P. polonicum	0.05	$2.4\ 10^9$
T. trachyspermus	0.5	$2.35\ 10^{10}$
C. spicifera	0.23	$2.4\ 10^8$
A. ustus	0.07	$7.6\ 10^8$
A. hiratsukae	0.37	$1.6\ 10^{10}$
A. niger	0.37	$6.2\ 10^9$
A. alternata	0.21	$3.6\ 10^8$
R. oryzae	0.4	$6.8\ 10^9$
C. cladosporioides	0.14	$5.8\ 10^9$
P. citreosulfuratum	0.12	$2.64\ 10^9$
P. commune	0.12	$1.6\ 10^{10}$
P. toxicarium	0.10	$3.4\ 10^9$
M. verrucosa	0.13	$3.2\ 10^9$
A. melanogenum	0.29	$3.6\ 10^9$

P. expansum	0.24	2.56 10^9
A. awamori	0.33	1.2 10^9
A. proliferans	0.16	2.4 10^9
P. griseofulvum	0.3	2.3 10^{10}
T. virens	0.8	1.6 10^{10}
A. welwitschiae	0.6	1.5$10^{10}$
P. lilacinum	0.3	1.9$10^{10}$
M. circinelloides	0.25	2.4$10^9$

Penicillium commune

On PDA and MEA medium the thallus is characterized by a medium apical growth (0.12 mm per day at 30°C), velvety appearance, green-blue or green-yellow (Fig. 6 A). The culture has an elevated structure in the center, with deep folds. There is exudate production on the surface of the colonies (visible with the binocular loupe). The reverse side of the colony is yellow to gray. Under the microscope, zygospores that do not exceed 50µm in diameter. Globular spores, smooth and hyaline. (Fig. 6 B).

The results of PCR and sequencing are presented in the Table 5 and Figure 7.

Table 7. Fungal colony apical growth (mm/h) of strains cultivated on different culture media and incubation at 30 ° C during 7 days.

Strains	Glucose	Sucrose	Starch	Casein	Tween	Phyticacid	Tannicacid	carboxymethylcellulose
P. expansum	0.33	0.27	0.13	0.29	0.34	0	0	0.2
G. Candidum	0.37	0.33	0.17	0.35	0.31	0.23	0	0.54
A. niger	0.33	0.34	0.20	0.23	0.17	0.27	0.31	1.6
P. crustosum	0.25	0.31	0.14	0.31	0.36	0	0	1.15
A. infectoria	0.22	0.24	0.19	0.26	0.16	0	0.27	0.4
Mucor fragilis	0.30	0.36	0.32	0.32	0.20	0.34	0	0.75
A. pseudoglaucus	0.19	0.22	0.17	0.22	0.17	0.18	0	0.7
T. assiutensis	0.33	0.34	0.12	0.28	0.18	0.15	0.35	0.55
C. spicifera	0.18	0.22	0.12	0.25	0	0	0	0.24
A. ustus	0.25	0.25	0.16	0.25	0	0	0.26	1.3
T. virens	0.41	0.48	0.20	0.41	0.41	0.19	0.25	1.9
A. awamori	0.16	0.15	0.52	0.15	0.52	0.52	0	0.4
A. alternata	0.13	0.12	0.18	0.11	0.17	0.28	0	0.61
R.oryzae	0.3	0.32	0.12	0.15	0	0	0.26	0.81
C.cladosporioides	0.21	0.23	0.13	0.25	0	0	0.24	0.2
P.citreosulfuratum	0.25	0.21	0.25	0.25	0.41	0.34	0.27	0.6
P. commune	0.19	0.19	0.14	0.41	0.18	0.15	0.52	0.45
F.oxysporum	0.19	0.21	0.15	0.3	0.2	0.25	0.16	0.7
Myceliophthoraverrucosa	0.12	0.11	0.19	0.12	0.17	0.17	0	0.57
A. tubingensis	0.18	0.22	0.11	0.25	0.11	0.10	0	0.4
P. polonicum	0.15	0.16	0.19	0.16	0.22	0.2	0	0.5
T. trachyspermus	0.11	0.009	0.14	0.09	0.11	0.13	0.08	0.4
A.hiratsukae	0.25	0.21	0.25	0.25	0.41	0.41	0.27	1.25
P.toxicarium	0.33	0.18	0.20	0.45	0.18	0.21	0.41	1.08
A. melanogenum	0.22	0.15	0.23	0.45	0.20	0.17	0.41	1.1
A. proliferans	0.28	0.28	0.14	0.25	0.15	0.18	0.25	0.76
P.griseofulvum	0.18	0.22	0.11	0.25	0.11	0.10	0.13	0.6
A. welwitschiae	0.29	0.25	0.18	0.28	0.22	0.21	0.31	0.42
P.lilacinum	0.22	0.15	0.23	0.45	0.20	0.17	0.41	1.1
M. circinelloides	0.19	0.21	0.14	0.26	0	0.22	0	0.35

Distribution of mesophilic filamentous fungi

The distribution of mesophilic strains according to their genera is given in Fig. 8. The isolates thus obtained mainly belonged to the genera *Aspergillus*, *Penicillium*, *Alternaria*, *Talaromyces*, *Geotrichum Cladosporium*, *Mucorale*, *Fusarium*, *Aureobasidium*, *Curvularia*, *Trichoderma*, *Purpureocillium*, *Rhizopus*, *Myceliophthora*. The percentage distribution of different species showed a predominance of *Aspergillus* (33%) and *Penicillium* (26%). The remainder were related to the following genera: *Alernaria*, *Talaromyces*(7%), *Geotrichum*,

Cladosporium, *Mucorale*(4%) and *Fusarium*, *Aureobasidium*, *Curvularia*, *Trichoderma*, *Purpureocillium*, *Rhizopus*, *Myceliophthora* (2%).The Same results were found by Roussos *et al.* (2006) who showed that mesophilic dominant mycoflora isolated from olives and olive cake belong to *Aspergillus* and *Penicillium* genera and the other isolated strains include *Geotrichum*, *Mucor*, and *Rhizopus*. Similarly, *Aspergillus* species were isolated from olives and olive fatty cakes by Belaiche (2001) and Leondopoulos *et al.* (2003). The previous reports also illustrate that *Aspergillus* species are the dominant filamentous fungi found on olives and olive products (Gracian and Arwalo, 1980).

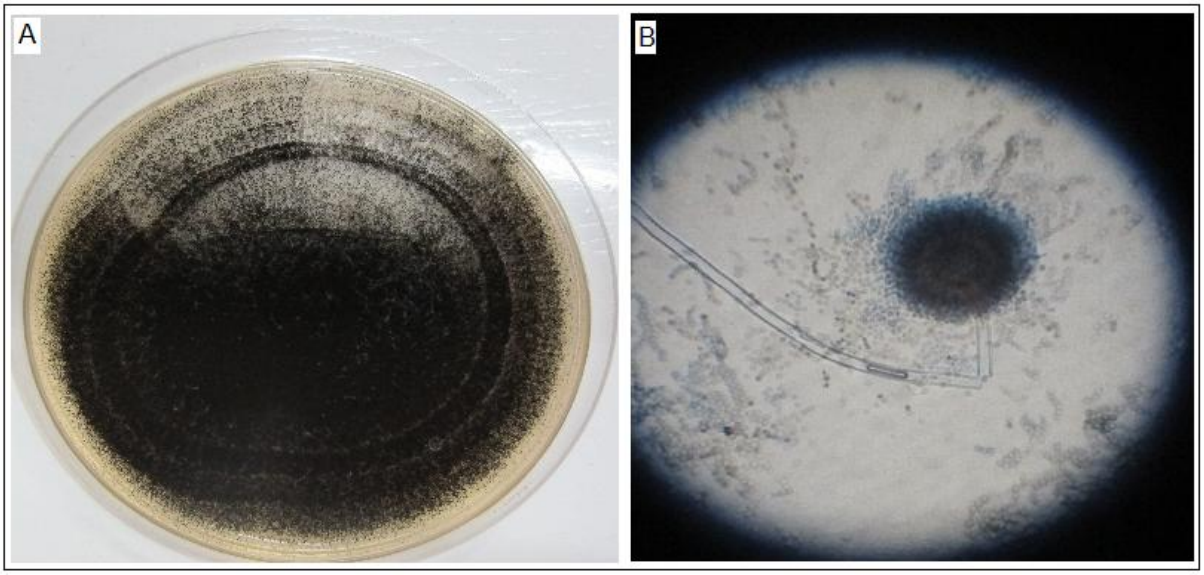

Fig. 2. *Aspergillus niger*on PDA medium at 30°C (A), Microscopic observation,x 400 (B).

GGGGCTTTCCGGGTCGGGTCTTTGGGCCACCTCCCATCCGTGTCTATTG
TACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCCGCCGGGGGGGCG
CCTCTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACACGAACACTG
TCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTT
CAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATG
CGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAA
CGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTC
ATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTC
CGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGA
GCGTATGGGGCTTTGTCACATGCTCTGTAGGATTGGCCGGCGCCTGCCG
ACGTTTTCCAACCATTCTTTCCAGGTTGACCTCGGATCAGGTAGGGATA
CCCGCTGAACTTAAGCATATCAATAAGCGGAGGAATTTTTTTTTTTTTT
TATTGGTGGGGAAGGAG

Fig. 3. Nucleotide sequence of the ITS region of the *Aspergillus niger* strain isolated from olive cake from the Zarzis region.

Table 2 shows the distribution of the different genera and species isolated from OMW and olive cake from different regions of Tunisia. Our results showed the presence of a very interesting fungal biodiversity. According to this result, the samples of OMW and olive cake from the southern region (Zarzis, Sfax and Chaal) contain a higher number of fungi than the northern and Sahel regions. For OMW, despite their toxic composition, we found a very important number of strains which exceeds the olive cake in all regions. Moreover, the acidic pH of olive mill wastes may be advantageous for this microbial group to out compete

bacteria. Filamentous fungi, such as *Aspergillus* and *Penicillium* spp., are common habitants of olive mill wastes; the same results were fund by (Millan *et al*., 2000,Aissam *et al*., 2007).Therefore, the members of the fungal genera *Acremonium, Alternaria, Aspergillus, Chalara, Fusarium, Lecythophora, Paecilomyces, Penicillium, Phoma, Phycomyces, Rhinocladiella,* and *Scopulariopsis* have been identified in OMW disposal ponds, possessing the ability to detoxify olive mill effluents (Millan *et al*., 2000).Also, it should be noted that the composition of our samples favors the presence of this biodiversity of fungi, in olive cake due to their composition, same results was fund by by Roussos *et al.* (2006) and Lamrani *et al.* (2006).

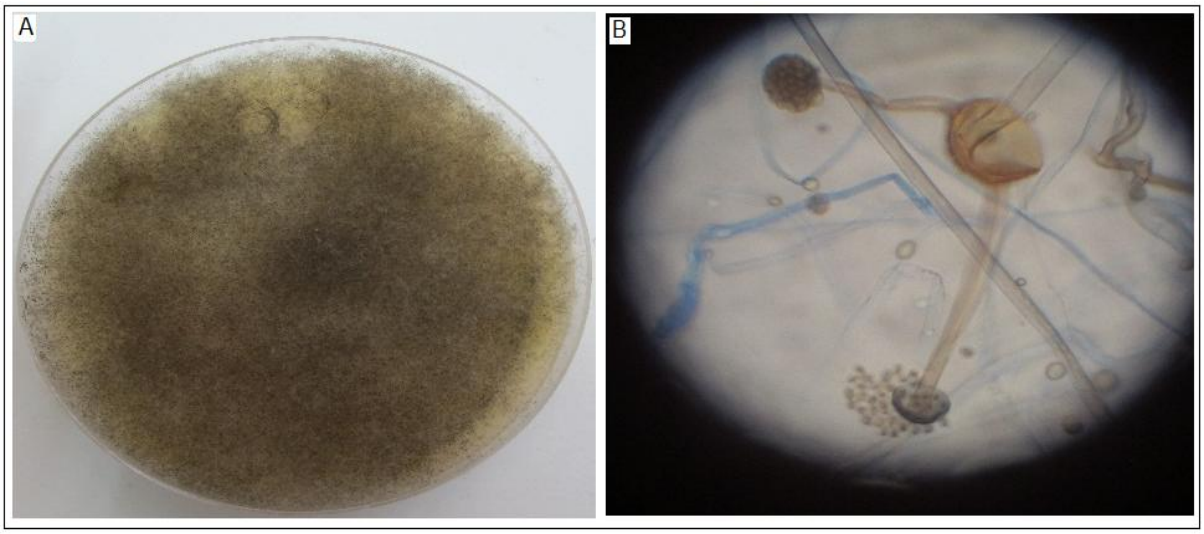

Fig. 4. *Rhizopus oryzae* on PDA medium at 30°C (A). Microscopic observation, x 400 (B).

TCGATCATAGTTTGAAAGTTACTGGATTATACTCTTGTACTTTACTTCC
TGGGCGAACCAAAAAAAAAAGATCCTGAGACCAGCGTAATATTCCTGCCT
AGCAAGCCAGACAGAAAATCACACACATTTTAGGTGCTCACTGTAATAA
AACAGCGATGCGACCCATCACCACATAAACAAATGTTATGTGTGGGTTT
GTGATGATACTGAAGCAGGCGTACTCTATAGAAAAACCATAGAGTGCAA
GCTGCGTTCAAAGACTCGATGATTCACTGAATATGCAATTCACACTAGT
TATCGCACTTTGCTACGTTCTTCATCGATGCGAGAACCAAGAGATCCAT
TGTTAAAAGTTGTTTTTTATTAAACTTTATAATACTGAATTTCTAGGTT
TATTATGAAGGGTGCTCCTGAAACCAGGAGTGGCATCGATCAAACCCCA
GATAGGTCTACCCATGACCAGTCTGAGTCTCTCAGCCAA

Fig. 5. Nucleotide sequence of the ITS region of the *Rhizopus oryzae*strain isolated from olive cake from the Jemmal region.

Physiological characterization

The measurement of apical growth indicates the capacity of filamentous fungi for superficial colonization on culture medium. This provides information on the kinetics of biomass production and for better understanding of the growth strategy of fungi in their natural habitat. The data on the apical growth and sporulation index of isolated strains are presented in Table 6.

The results obtained indicate that the isolated strains showed variable apical growth rate and sporulation index at a constant incubation temperature. The comparison of sporulation and apical growth averages for different species revealed an interval ranging from 2.410^{8} to 2.3510^{10} spores /cm² and from 0.05 to 0.8

mm/h respectively. *Talaromyces trachyspermus, Penicillium griseofulvum, Trichoderma virens, Purpureocillium lilacinum, Aspergillus welwitschiae* showed the highest sporulation index and *Trichoderma virens, Fusarium oxysporum, Talaromyces trachyspermus, R. oryzae* showed the highest apical growth.

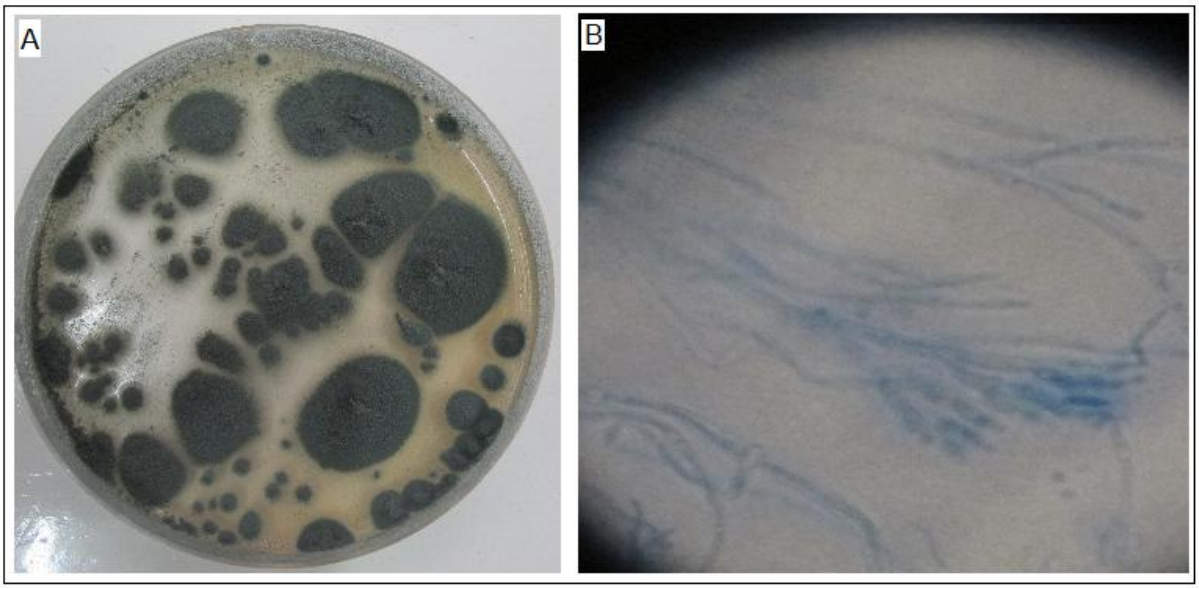

Fig. 6. *Penicillium commune*on PDA medium at 30°C (A). Microscopic observation of strain, x 400 (B).

CGAGGTCACCTGGATAAAAATTTGGGTTGATCGGCAAGCGCCGGCCGGGCCTACAGAGC
GGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGC
CCGTCCCCCGGAGATCGGGGGACGGGGCCCAACACACAAGCCGGGCTTGAGGGCAGCAA
TGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGAC
TCGATGATTCACTGAATTTGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTCAT
CGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAAATAATTTATATTTTCACTCA
GACTTCAATCTTCAGACAGAGTTCGAGGGTGTCTTCGGCGGGCGCGGGCCCGGGGGCGT
AAGCCCCCCGGCGGCCAGTTAAGGCGGGCCCGCCGAAGCAACAAGGTAAAATAAACACG
GGTGGGAG

Fig. 7. Nucleotide sequence of the ITS region of the *Penicillium commune* strain isolated from OMW from the Châal region.

Biochemical characterization

Fig. 9 shows the percentage of strains degrading the different carbon sources. Thus, it can be noted that starch, sucrose, glucose, casein and carboxymethylcellulose have been used by all the strains, while tannic acid is the least used by the strains with a percentage of 67%. It can be noted also that there are the percentages that differ according to the species and even according to the strain the same results was fund by Lamrani *et al.* (2006) which have also shown that the use of different sources of carbon depends on the production of the enzyme degrading the corresponding substrate. The measurement of the apical growth of different species according to different carbon sources (Table 7) showed that all fungi can degrade a variety of carbon sources at 30 ° C.

It can be noted that the apical growth varies according to the species and even according to the

strain. On average all strains use glucose, sucrose, casein and starch, suggesting that these species can produce the extracellular proteases and amylases. *T. virens* is the only strain that has shown good apical growth on all medium, so it is able to use all sources of carbon. For *P. expansium* and *P. crustosum* they do not degrade the phytase, however sometimes only mycelium is found without any sporulation.

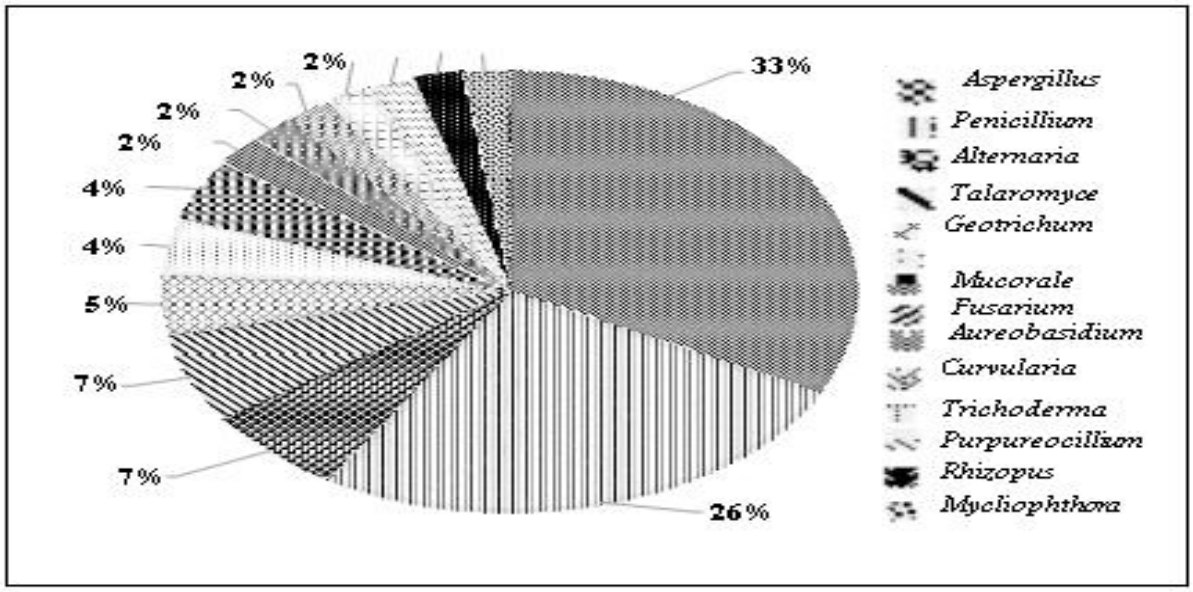

Fig. 8. Proportions (%) of different fungi isolated from olive mill waste water and olive cake from different regions of Tunisia.

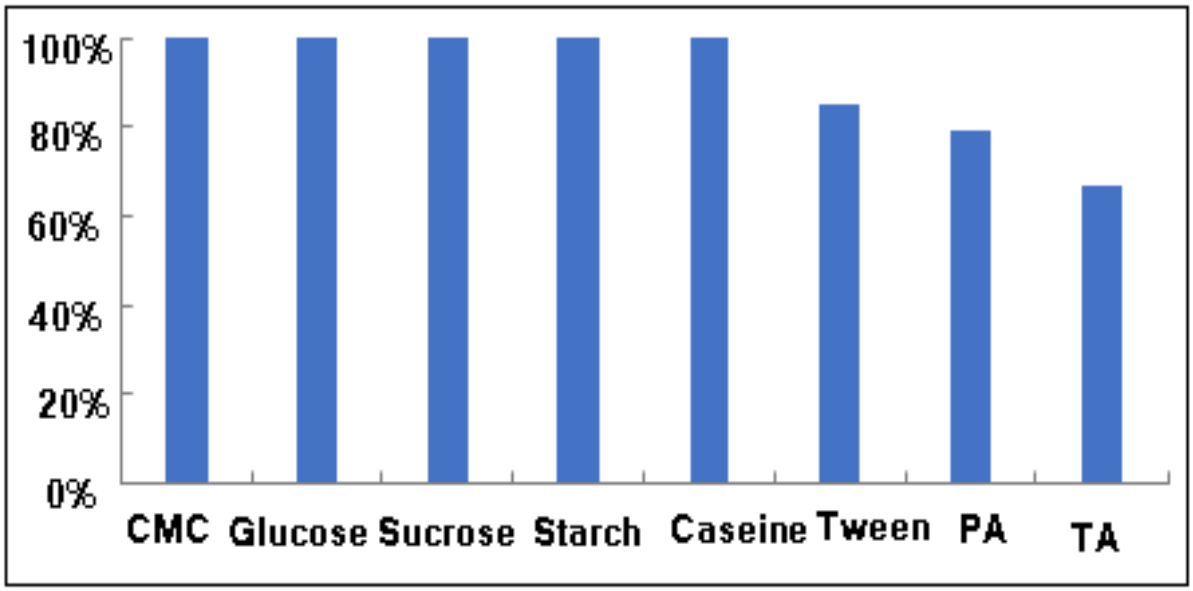

Fig. 9. Percentage of strains degrading different carbon sources.

This was also reported by Tansey (1971) and Lamrani *et al.* (2006) who showed that *Rh pusillus* and *Rhmiehei* do not degrade the cellulose, but sometimes only mycelium is found without any sporulation. Nevertheless, these two species degrade Tween 80 well, so may be able to produce extracellular lipases at 30°C.The degradation of tannic acid makes it possible to differentiate between *P. expansium* and *P. crustosum*. For phytic acid, *A. awamori* and *R. stolonifera* can use well this source. For tannic acid, *Purpureocillium lilacinum* and *P. common* exhibits good apical growth. The results obtained show that

our fungi collection can produce several extracellular enzymes such as Tannic acid, phytic acid, amylase, cellulase .... Therefore, the isolated strains are able to hydrolyze and assimilate different substrates as unique carbon source (depending on the species) after incubation at30°C.

Conclusion

This work is an original contribution by studying the biodiversity of filamentous fungi isolated from by-products of the olive tree (OMW and olive cake) taken from the various olive mill located in the main olive growing regions of Tunisia (North, South and Sahel). A collection of 47 mesophilic strains has been constituted and conserved. The different mills of Tunisia have, therefore, proved to be one of the particular biotopes, with an interesting fungal biodiversity. All the isolated strains have the following enzymatic activities: amylolytic, proteolytic and cellulolytic. These results suggest that our fungi collection displays an important biotechnological potential, thereby the isolated strains can produce several extracellular enzymes of great interest for biotechnology and industry such as amylase, phytase, protease and cellulase which represent high added value products.

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